

Exogenous trehalose promotes non-structural carbohydrate accumulation and induces chemical detoxification and stress response proteins in *Arabidopsis thaliana* grown in liquid culture

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Abstract

Trehalose is a non-reducing disaccharide that functions as a storage carbohydrate and osmoprotectant in yeast, fungi and certain insects. Endogenous trehalose also is present in trace amounts in flowering plants and metabolites derived from trehalose were necessary for embryo development. In contrast to its role in endogenous metabolism, exogenous trehalose is toxic to higher plants as evidenced by a severe inhibition of root and cotyledon growth. In the current study, 30 mM trehalose or sterile water was added to 2-week-old liquid cultures containing *Arabidopsis thaliana* (L.) Henyeh seedlings. After 12 h of exogenous trehalose treatment sucrose and trehalose were increased 3.2- and 145-fold, respectively, compared to the controls. These were the only metabolites measured in this study that responded to trehalose treatment at this time point. Starch was increased and ATP levels were decreased in *Arabidopsis* samples after 1 day of treatment with 30 mM trehalose. Conversely, glutamate, glutamine, hexoses and chlorophyll were unaffected by trehalose treatment. Two-dimensional gel electrophoresis revealed that for whole *Arabidopsis* seedlings, nine proteins were altered by 12 h of trehalose treatment relative to the controls. Seven of the nine polypeptides were successfully identified by sequence analysis. Four of the identified proteins induced by trehalose treatment, Phi glutathione *S*-transferase 2 (*AtGSTF2*), flavin mononucleotide-binding flavodoxin-like quinone reductase 1 (*FQR1*), cytosolic dehydroascorbate reductase 1 (*DHAR1*) and *S*-adenosylmethionine synthetase 2 (*SAMS2*), were involved in either detoxification or stress responses. The above results indicated that feeding exogenous trehalose to liquid cultures of *Arabidopsis* seedlings rapidly altered disaccharide levels and induced detoxification and stress response proteins. These findings supported the suggestion that exogenous trehalose functioned as an elicitor of genes involved in biotic and abiotic stress.

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Keywords: Proteomics; Plant stress; Non-structural carbohydrates; Sugar signaling; Gene expression

1. Introduction

Trehalose (1,1- α -D-glucopyranosyl α -D-glucopyranoside) is a non-reducing disaccharide consisting of two glucose residues joined by an α -1-1 bond. Most living organisms manufacture trehalose [1] and in yeast and certain fungi trehalose functions as the primary storage carbohydrate and as a stress protectant [2–4]. Trehalose is present in trace amounts in almost all flowering plants, so that the role of trehalose in higher and lower plants likely differs [2].

Abbreviations: MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; T6P, trehalose 6-phosphate; GST, glutathione *S*-transferase; GSH, glutathione; DHAR, dehydroascorbate reductase; PDI, protein disulfide isomerase; SAMS, *S*-adenosylmethionine synthetase; SAM, *S*-adenosylmethionine; FQR, flavodoxin-like quinone reductase; TPI, triose-phosphate isomerase; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase

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Trehalose is synthesized from glucose-6-phosphate and uridine-5-diphosphoglucose by the sequential actions of trehalose 6-phosphate synthase and trehalose 6-phosphate phosphatase, generating trehalose-6-phosphate (T6P) and trehalose, respectively (reviewed in 5). The hydrolysis of trehalose occurs by the action of α -trehalase, a specific α -glucosidase that generates two glucose moieties [3]. Over-expressing genes responsible for trehalose biosynthesis in higher plants confers resistance to abiotic stress and also alters plant growth and development [4,6].

Feeding exogenous trehalose to higher plants altered carbohydrate metabolism. Wingler et al. [7] reported that starch levels were increased and sucrose levels were reduced in shoots of *Arabidopsis* seedlings grown on agar plates containing trehalose for 10 days. Leaf expansion and root elongation in *Arabidopsis* were drastically inhibited during growth on media containing 10–30 mM trehalose [3,7,8]. However, the inhibition of seedling growth in *Arabidopsis* by trehalose was partially reversed by the presence of sucrose or of various hexoses. Recent evidence indicated that T6P was synthesized by *Arabidopsis* in response to feeding trehalose and sucrose. The experimental evidence indicated that T6P rather than trehalose itself was responsible for altering the concentrations of various photosynthetic and glycolytic intermediates in *Arabidopsis* seedlings [9]. Also, T6P was implicated in the growth inhibition that occurred in response to feeding trehalose. These findings were consistent with previous results showing that T6P was an essential factor for embryo maturation in *Arabidopsis* [10] and with the suggestion that T6P may regulate carbohydrate metabolism in plants [11].

It is clear from the above discussion that exogenous trehalose treatment altered metabolite levels in various higher plants, but these were invariably longer-term studies that occurred over 1–8 weeks [7,9]. We hypothesized that altered metabolite levels in higher plants were, in part, due to developmental changes induced by trehalose treatment. Also, changes of transcript abundance in response to signaling events can be extremely rapid [12]. Therefore, a primary goal of this study was to examine metabolite changes that occurred over the 1st few hours of trehalose treatment.

Like several other metabolizable sugars [13,14], feeding trehalose exogenously to higher plants altered the activity of various enzymes. Müller et al. [15] reported that feeding trehalose to the cut ends of excised barley leaves induced the expression of sucrose:fructan-6-fructosyl-transferase, an enzyme involved in fructan synthesis. Similarly, the large subunit (*ApL3*) of ADP-glucose pyrophosphorylase (AGPase) in *Arabidopsis* was induced by trehalose feeding [15,16]. Changes of AGPase activity in response to trehalose treatment were correlated with increased leaf starch levels. Exogenous trehalose also increased sucrose synthase and alkaline invertase activities but concomitantly reduced acid invertase activity and sucrose levels in soybean roots [17]. The regulation of gene expression by carbohydrates is now recognized as an important adaptive mechanism in yeasts,

mammals and higher plants [13,14]. Attaining a balance between C production and utilization is particularly important during periods of environmental or nutritional stress.

Müller et al. [17] speculated that symbionts, plant pathogens and other microbionts released trehalose to redirect carbohydrate metabolism in host tissues, and that α -trehalase produced by plants may function defensively to remove the externally produced disaccharide. In support of this suggestion, trehalose applications induced two stress response enzymes, phenylalanine ammonia-lyase and peroxidase, in wheat leaves and multiple applications of trehalose conferred resistance to powdery mildew [18]. Also, a transcription profiling study revealed that increased T6P levels were associated with six stress responses genes, including three stress related protein kinases [8]. Taken together the above findings indicated that trehalose feeding modified protein levels of higher plants and that this was possibly mediated by T6P.

The main objective of the current study was to investigate metabolites and proteins in *Arabidopsis* that were altered during the first few hours of treatment with exogenous trehalose. To accomplish this objective we used seedlings grown in liquid culture and a proteomics approach involving 2-D gel electrophoresis and polypeptide analysis via MALDI-TOF. Proteomics overcomes the main shortcoming of transcriptional profiling, which does not quantify the final end products of gene expression [19]. Also, there is a poor correlation between mRNA levels and protein abundance due to differences in turnover rates [20]. Current findings were that exposure to trehalose for as little as 12 h induced systemic changes in *Arabidopsis* that altered metabolite levels and induced four known stress response and chemical detoxification proteins.

2. Materials and methods

2.1. Plant materials

Arabidopsis seeds of the Columbia ecotype were sterilized with one-third strength commercial bleach for 10 min and were then rinsed 5 times with sterile, deionized water. Approximately 50 seeds were transferred to sterile 250 ml Erlenmeyer flasks containing 50 ml of 1 \times strength Murashige-Skoog (MS) basal salts medium supplemented with 1 \times strength vitamin B5, 1% (w/v) sucrose and 0.1 mM MES-NaOH (pH 5.7) buffer. Seedlings were grown in controlled environment chambers under continuous fluorescent light providing $150 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD measured at flask height. Air temperature was a constant 22 °C and the flasks were agitated continuously at 100 rpm on a rotary shaker. After 2 weeks growth sufficient amounts of a filter-sterilized trehalose solution (Sigma, St. Louis, MO) were added to one-half of the flasks to yield a final concentration of 30 mM (i.e., 1% w/v). An equivalent

amount of sterile, deionized water was added to the control flasks. Seedlings were harvested at 0, 6, 12, and 24 h after treatment with trehalose commenced. Harvested seedlings were poured onto a stainless steel sieve, rinsed several times with deionized water and were immediately frozen in liquid N₂ to stop metabolism. Samples were stored at -80°C for up to 1 month if necessary.

2.2. Biochemical analyses

Whole seedlings were ground to a fine powder using a mortar and pestle filled with liquid N₂. Frozen samples (0.25 g FW) were then homogenized with a ground glass tissue homogenizer at 4°C in 2 ml of extraction buffer (methanol, chloroform, and 52 mM aqueous Tris⁺ base (63:26:11)). Homogenates were collected in 15 ml centrifuge tubes and were spun at $3000 \times g$ for 10 min at 4°C in a model J2-HS centrifuge (Beckman Instr., Palo Alto, CA, USA). Supernatants were collected in a separate set of centrifuge tubes and the pellets were washed once or twice with 1 ml of 80% methanol with centrifuge steps as described above. The combined supernatants were partitioned with CHCl₃:H₂O (2:1) and the aqueous-alcohol fractions were evaporated to a minimum volume under a stream of N₂ gas at 37°C . The concentrated extracts were reconstituted in 1 ml of deionized H₂O. Metabolites were measured essentially as described earlier [21]. Pellets were gelatinized in 2 ml boiling water and starch was measured as glucose generated by the enzymic action of α -amylase and amyloglucosidase. Sucrose, glucose, fructose, ATP, glutamate and glutamine were measured via coupled enzyme assays using the aqueous fraction. Means were for three samples and the experiment was replicated once ($n = 6$). Significant differences were estimated using Student's *t*-test.

Trehalose was measured using gas chromatography coupled to a mass selective detector as described by Roessner et al. [22]. Frozen plant material (0.1 g FW) was extracted at 0°C with 1.4 ml ice-cold methanol using a ground-glass tissue homogenizer. The homogenates were heated for 15 min in a 70°C in a water bath and then diluted with an equal volume of deionized H₂O. The extracts were centrifuged at room temperature for 15 min at $12,000 \times g$ and an aliquot of the supernatant of each sample was evaporated under a stream of N₂ gas at 37°C . The dried samples were derivatized for 30 min at 37°C with 80 μl of MSTFA (*N*-methyl-*N*-(trimethylsilyl)fluoroacetamide) and ribitol served as an internal standard. Separations were performed on a Hewlett Packard model 6890 Gas Chromatography system using a 0.25 mm by 30 m SPB-50 column (Supelco, Bellefont, PA, USA). Standard curves were prepared with known amounts of trehalose.

2.3. Two-dimensional (2D)-gel electrophoresis

Whole seedlings grown in liquid culture were ground to a fine powder with liquid N₂ as described above. Equal

amounts of tissue after 6 and 12 h of trehalose treatment were combined and total protein was extracted according to a modified method developed for Arabidopsis by Dr. H. Theillement (<http://www.pierroton.inra.fr/genetics/2D/Proteomevert/Protocols/protocole.geneve.html>). Samples were collected at two time points because the time course for protein changes in response to trehalose treatment was unknown. Samples (0.5 g FW) were extracted in a ground glass tissue homogenizer with 5 ml of precipitation solution (10% (w/v) TCA in acetone cooled to -20°C containing 0.07% (v/v) 2-mercaptoethanol). The glass homogenizer was rinsed with an additional 5 ml precipitation solution and the homogenates were combined in a 35 ml polypropylene centrifuge tube. The samples were incubated at -20°C for 1 h and were then centrifuged at $27,000 \times g$ for 15 min at -20°C . The supernatants were discarded and the pellets were re-suspended in 10 ml of rinse solution (acetone cooled to -20°C containing 0.07% (v/v) 2-mercaptoethanol). The re-suspended pellets were incubated at -20°C for 1 h and centrifuged as described above. Wash steps were repeated 2–3 times until the pellets were colorless. The washed pellets were vacuum dried for 30 min and total proteins were dissolved in 1 ml of re-solubilization solution (9 M urea, 1% (w/v) CHAPS, 1% DTT, 1% Biolytes pH 3–10). Re-suspended samples were placed in a sonicator bath at room temperature for 5 min. Undissolved material was removed by centrifugation for 5 min at $27,000 \times g$ at room temperature and the resultant supernatants were stored at -20°C . An aliquot of the re-solubilized total protein preparation was precipitated with an equal volume of 10% TCA, and after centrifugation the pellets were dissolved in 1 N NaOH. Protein concentrations were then measured using a Coomassie blue dye-binding assay.

Except where noted all equipment and supplies were from Bio-Rad Corp., Hercules, CA, USA. Re-solubilized proteins (0.1 mg for Silver staining and 1 mg for Coomassie Brilliant Blue G-250 staining) for isoelectric focusing were diluted with sufficient rehydration buffer (8.3 M urea, 2% (w/v) CHAPS, 2 mM tributyl phosphine, 0.5% (v/v) Biolytes pH 3–10) to give a total volume of 0.25 ml. Samples were applied to 18 cm immobilized pH gradient (IPG) strips (Immobiline DryStrips (pH 4–7), Amersham Pharmacia Biotech, Piscataway, NJ, USA) and were allowed to rehydrate passively at 20°C for 12 h. Isoelectric focusing was performed with a Protean IEF system at 20°C according to a preset method with stage 1 at 250 V for 15 min, stage 2 with linear ramping from 250 to 10^4 V over 30 min, stage 3 at 10^4 V for 60×10^3 V h and, if necessary, stage 4 at 500 V until focusing was terminated. A maximum current of 50 μA per IPG strip was used to prevent overheating. Focused strips were used for electrophoresis immediately or were stored at -80°C .

The focused IPG strips were incubated sequentially with equilibration buffer I (6 M urea, 2% (w/v) SDS, 0.375 M Tris-HCl, pH 8.80, 20% (v/v) glycerol, a trace of bromophenol blue and 2% DTT) and equilibration buffer

II (6 M urea, 2% (w/v) SDS, 0.375 M Tris–HCl, pH 8.80, 20% (v/v) glycerol, a trace of bromophenol blue and 2.75% (w/v) iodoacetamide) for 15 min each. The equilibrated strips were quickly sealed on top of 20 cm × 20 cm 12% denaturing gels with 0.5% agarose in electrode buffer Laemmli (1970). Electrophoresis of up to six gels at a time was performed in a PROTEAN II apparatus using 15–30 mA per gel for 14–16 h. Image analysis was performed on silver stained gels using a FluorS Max Multimager and PDQuest software. Imaging results were analyzed using three separate replications of the 2D-gel electrophoresis experiments. A second set of gels was stained with Coomassie Brilliant Blue G-250 and selected protein spots were excised for polypeptide analysis. Destaining and tryptic digestion were performed according to a protocol provided by the University of Minnesota, Mass Spectrometry Facility (http://www.cbs.umn.edu/mass_spec). Peptide sequences were determined by MALDI-TOF mass spectrometry performed at the same facility. Protein identities were based on deduced nucleotide sequences obtained with a web based software program (MASCOT http://www.matrix-science.com/cgi/index.pl?page=/search_form_select.html). Protein identities were then confirmed by comparing the actual and deduced Mr and pI of each spot.

3. Results

3.1. Effects of exogenous trehalose on plant growth

Attempts to grow *Arabidopsis* in liquid culture with MS basal salts generally were unsatisfactory. Approximately 50 *Arabidopsis* seeds grown for 2 weeks with 50 ml of MS

medium at 22 °C and at $150 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD yielded about 10 mg fresh weight (FW) of largely achlorotic plant material (data not shown). However, over 1 g FW of tissue was obtained under the same growth conditions if the basal salts medium was supplemented with 1% (w/v) sucrose or glucose. Fructose and glutamate also were used successfully but the total biomass yield per flask was less than that with either sucrose or glucose (data not shown). Seedlings treated with 30 mM trehalose were green, healthy and were able to flower after 4 weeks growth in liquid culture, whereas the untreated control seedlings grown on MS basal salts with 1% sucrose were completely chlorotic and were no longer viable at this time (data not shown). *Arabidopsis* seedlings grown on MS media in liquid culture with 1% sucrose did not display the drastic inhibition of root growth by 10–30 mM trehalose that was observed after 2 weeks of growth on MS agar plates [3,7]. However, the growth of 2-week-old *Arabidopsis* seedlings was stunted when of 30 mM trehalose was added to the culture medium and never reached that of control seedlings grown with 1% sucrose (data not shown).

3.2. Effects of exogenous trehalose on metabolite levels

Changes of metabolite levels in 14-day-old *Arabidopsis* seedlings grown in liquid culture in response to 30 mM trehalose are shown in Table 1. Sucrose levels in *Arabidopsis* samples increased 3.2-fold in response to 12 h of treatment with exogenous trehalose. Trehalose concentrations in *Arabidopsis* seedlings increased from about 2 to $290 \mu\text{g g}^{-1}$ FW within 12 h after 30 mM trehalose was added to the liquid culture medium. These were the only two metabolites that differed significantly after 12 h of trehalose

Table 1
Changes of metabolite levels in *Arabidopsis* seedlings in response to exogenous 30 mM trehalose

Compound	Treatment	Time		
		0-h	Plus 12 h	Plus 24 h
Glucose	Control	0.12 ± 0.02	0.14 ± 0.02	0.14 ± 0.03
	+30 mM Trehalose		0.19 ± 0.01	0.10 ± 0.02
Fructose	Control	0.23 ± 0.03	0.23 ± 0.03	0.22 ± 0.05
	+30 mM Trehalose		0.27 ± 0.01	0.15 ± 0.02
Starch	Control	0.75 ± 0.17	1.90 ± 0.37	1.49 ± 0.27
	+30 mM Trehalose		1.48 ± 0.21	2.45 ± 0.10*
Sucrose	Control	0.13 ± 0.03	0.19 ± 0.02	0.19 ± 0.04
	+30 mM Trehalose		0.79 ± 0.05**	0.93 ± 0.05**
Glutamate	Control	0.25 ± 0.02	0.19 ± 0.05	0.24 ± 0.06
	+30 mM Trehalose		0.28 ± 0.02	0.37 ± 0.03
Glutamine	Control	3.47 ± 0.47	3.00 ± 0.26	2.75 ± 0.50
	+30 mM Trehalose		3.33 ± 0.15	2.97 ± 0.38
ATP	Control	0.09 ± 0.01	0.18 ± 0.02	0.13 ± 0.01
	+30 mM Trehalose		0.15 ± 0.01	0.05 ± 0.01*
Trehalose	Control	ND	<0.01	ND
	+30 mM Trehalose		0.29 ± 0.02**	ND

Sterile trehalose or deionized H₂O (control samples) was added to 14-day-old liquid cultures and plant material was harvested at indicated times by filtering. Sample means are in mg g⁻¹ FW and are for *n* = 6. ND: not determined.

* Significant differences of *P* ≤ 0.05.

** Significant differences of *P* ≤ 0.01.

treatment. However, 24 h after the start of treatment transitory starch levels were 64% greater in the trehalose treated samples than in the controls. Both sucrose and starch continued to accumulate in the trehalose treated samples. After 3 days of treatment with trehalose, starch and sucrose were 3.0- and 6.5-fold greater, respectively, in the trehalose treated samples than in the controls (data not shown). Glucose, fructose, glutamate and glutamine levels were not significantly different between the trehalose treated samples and the controls over the first day of trehalose treatment. Because the plant material in this study was grown in liquid culture, glutamine levels were about 15 times greater than that of glutamate. Also, total chlorophyll (a + b), the chlorophyll a/b ratio and carotenoids (x + c) were measured in this study but treatment with trehalose for 24 h had no effects these pigments (data not shown). ATP levels in *Arabidopsis* seedlings were similar in the controls and treated samples after 12 h of treatment with 30 mM trehalose. However, ATP levels were 74% lower in the treated samples compared to the controls 24 h after trehalose treatments were initiated. Based on results of the gas chromatography–mass spectrometry procedure various organic acids, i.e., lactic, pyruvic, fumaric, malic, succinic and citric, were unchanged in *Arabidopsis* seedlings after 12 h of trehalose treatment (data not shown).

3.3. Two-dimensional gel electrophoresis

Total proteins were extracted from intact 2-week-old *Arabidopsis* seedlings that were grown in liquid culture as

described above. Samples were harvested after a subsequent 6 or 12 h of treatment with 30 mM trehalose and then these samples were combined for analysis by 2D-gel electrophoresis. Approximately 400 major spots were compared between trehalose treated and control samples and a representative image of a silver stained gel is shown in Fig. 1. As indicated directly on the figure and in the side panel, nine protein spots were detected that showed altered expression patterns in response to the 30 mM exogenous trehalose treatment. Relative to the control samples eight protein spots were increased and one spot was decreased in the trehalose treated tissue compared to the controls. Note that these findings were based upon mean differences of three replicate experiments. Sequence analyses of the nine protein spots that were altered by trehalose treatment were performed by MALDI-TOF mass spectrometry. Seven polypeptides were successfully identified by sequence analysis and comparison with the *Arabidopsis* genome (Table 2). Based on changes in pixel densities, specific proteins in this study were increased from 73 to 501% by trehalose treatment. Exogenous trehalose treatment induced Phi glutathione *S*-transferase 2 (*AtGSTF2*), an enzyme that forms conjugates between glutathione (GSH) and xenobiotic substrates; cytosolic GSH-dependent dehydroascorbate reductase 1 (*AtDHAR1*), an ascorbate synthesizing enzyme in chloroplasts; protein disulfide isomerase (PDI) family, which are enzymes involved in the formation of protein disulfide bonds; *S*-adenosylmethionine synthetase 2 (*SAMS2*), a multifunctional enzyme; a flavin mononucleotide binding flavodoxin-like quinone reductase 1 (*FQR1*),

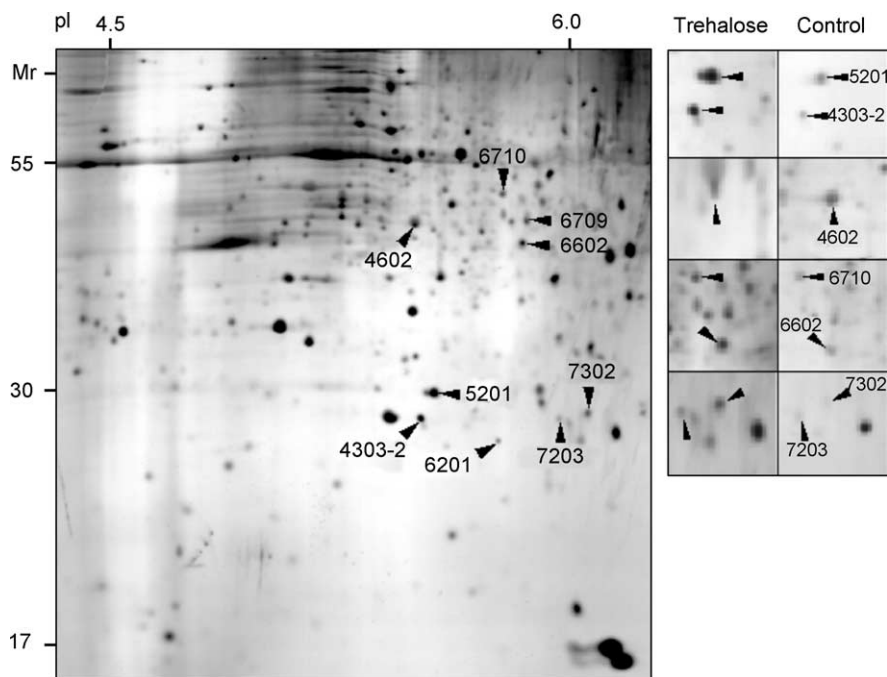


Fig. 1. A representative digital image of a silver-stained gel of total soluble proteins obtained from 2-week-old *Arabidopsis* seedlings grown in liquid culture. Numbered spots denote proteins altered after 6–12 h treatment with 30 mM trehalose. Images of seven identified polypeptides from treated and control samples are shown in a sidebar. Isoelectric point (pI) and molecular mass (kD) are indicated in the horizontal and vertical dimensions, respectively.

Table 2

Identities of seven specific polypeptides in *Arabidopsis* that changed abundance in response to 30 mM exogenous trehalose

No.	Spot no.	Masses (kD)	pI	Identity	Locus no.	Relative abundance ^a
1	4303-2	23.6	5.39	GSH-dependent dehydroascorbate reductase (DHAR1)	At1g19570	29 ^b
2	4602	52.0	5.87	Rubisco activase	At2g39730	−42 ± 8
3	5201	27.2	5.56	cytosolic triose phosphate isomerase	AL132975	141 ± 51
4	6602	39.5	5.80	Protein disulfide isomerase family	At2g47470	93 ± 28
5	6710	43.2	5.67	S-adenosylmethionine synthase 2 (SAMS2)	At4g01850	94 ± 57
6	7203	21.8	5.96	Flavin mononucleotide (FMN)-binding flavodoxin-like quinone reductase (FQR1)	At5g54500	318 ± 239
7	7302	24.0	6.08	Phi Glutathione S-transferase 2 (GSTF2)	At4g02520	193 ± 117

The molecular mass (kD) and isoelectric point (pI) of candidate proteins are shown.

^a Relative abundance was obtained from pixel densities of control and treated samples using silver stained gel images.

^b Average of two spots.

which reduces quinones and semiquinones; and cytosolic triose-phosphate isomerase (TPI), an enzyme that catalyzes the isomerization of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate in carbohydrate metabolism. The only protein that was repressed by trehalose was Rubisco activase, a chloroplast protein involved in the carbamylation of Rubisco to generate a catalytically active form of the enzyme.

4. Discussion

Prior studies [7,9] demonstrated that exogenous trehalose altered both the growth and development of *Arabidopsis*. According to Fritzius et al. [16] root lengths of *Arabidopsis* were decreased up to 80% by exogenous trehalose in comparison with control plants. Also, the over-expression of bacterial and yeast genes involved in trehalose metabolism in higher plants induced phenotypic changes in various transformant lines [2,5,9]. Unlike yeast or fungi, trehalose is present in trace amounts in higher plants [4,6]. Based on the above evidence, it was concluded that trehalose, or possibly metabolites derived from trehalose, likely functioned as elicitors or as a signaling molecules in higher plants [2,5,18]. The molecular mechanisms involved in trehalose sensing in higher plants are unknown. However, trehalose is a regulator of glycolytic flux in yeast and T6P inhibited hexokinase activity in this organism [23]. Preliminary results indicated that, unlike the situation in yeast, T6P was not an inhibitor of hexokinase activity in *Arabidopsis* [10].

According to earlier reports [7,8,16], trehalose altered levels of various metabolites in *Arabidopsis* seedlings grown on agar plates for several days to weeks. In the current study, sucrose and trehalose were the only two metabolites that increased in concentration after 12 h of exposure to 30 mM exogenous trehalose using *Arabidopsis* seedlings grown in liquid culture. Note that trehalose accumulated in *Arabidopsis* shoots grown on agar plates for 8 weeks containing 10–25 mM trehalose [3]. This finding suggested that *Arabidopsis* seedlings acquired trehalose from the medium and transported it from roots to shoots. However, because *Arabidopsis* was grown in liquid culture in the current study,

we cannot exclude the possibility that the buildup of trehalose observed here was partly due to external trehalose that was not completely removed by washing. In contrast to current findings Wingler et al. [7] reported that sucrose concentrations decreased in *Arabidopsis* shoots when seedlings were grown on solid medium containing trehalose in the absence of exogenous sucrose. This difference may be due to the fact that whole seedlings were analyzed in the current study. Also, the expression of the sucrose transporter gene, *AtSUC2*, was induced by both exogenous trehalose and sucrose in a prior study [7]. This could potentially explain the trehalose dependent accumulation of sucrose from the liquid medium observed in the present study.

In agreement with prior results [7] the allocation of C to starch was increased in *Arabidopsis* plants treated with trehalose. However, the earliest time point this was detected was 24 h after trehalose treatments were initiated. Unlike the disaccharides discussed above, glucose and fructose levels in *Arabidopsis* seedlings were unaffected by exogenous trehalose. Therefore, changes of plant growth and morphology in response to trehalose were not attributable to glucose moieties derived from trehalose cleavage [7,16]. Glutamate, glutamine, various organic acids and chlorophyll levels also were unaffected by exogenous trehalose treatment in the current study. This finding suggested that trehalose did not have pronounced effects on nitrogenous constituents of *Arabidopsis* seedlings [24]. In apparent contrast to this conclusion ATP levels were significantly lower in treated compared to control seedlings after 24 h exposure to 30 mM trehalose.

It is generally agreed that trehalose or metabolites related to trehalose function in sugar sensing and can modulate plant gene expression [14]. Trehalose treatment specifically increased transcripts of the large subunit (*ApL3*) of AGPase in *Arabidopsis* [7], the activities of sucrose synthase and alkaline invertase activities in soybean roots [17] and two stress response enzymes, phenylalanine ammonia-lyase and peroxidase, in wheat leaves [18]. Also, Schluepmann et al. [8] identified six stress response genes in *Arabidopsis* that clustered with T6P levels in a transcription profiling study. Note that these authors reported that T6P levels were increased by feeding trehalose to *Arabidopsis* seedlings

grown in liquid culture. Results of the current study indicated that the expression of an additional nine proteins was altered by exogenous trehalose treatment. Seven of the nine proteins in *Arabidopsis* that responded to exogenous trehalose treatment were identified by 2-dimensional gel electrophoresis. (1) *AtGSTF2* is a membrane associated detoxifying enzyme that was induced by ethylene, salicylic acid, auxin and by pathogen attack [25,26]. *AtGSTF2* also showed glutathione peroxidase activity with linoleic acid hydroperoxide as a substrate; (2) *AtDHAR1* from *Arabidopsis* shares 65% identity and 77% similarity in amino acid sequences with cytosolic DHAR1 of *Oryza sativa* [27]. DHAR1 reduces dehydroascorbate to ascorbate in chloroplasts, using reduced glutathione as an electron donor [28]. Ascorbate functions as an antioxidant and is a substrate for ascorbate peroxidase or violaxanthin de-epoxidase in chloroplasts [29]. These latter enzymes protect plants from oxidative stress and excess light energy by maintaining the xanthophyll cycle and scavenging reactive oxygen species; (3) PDI catalyzes disulfide exchange reactions resulting in the formation of cysteinyl bonds. Multifunctional roles have been reported for this protein that include, serving as the β -subunit of prolyl-4-hydroxylase [30]; as a glycosylation site binding protein [3,31]; as a component of the microsomal triglyceride transfer protein complex [32]; as a form-I phosphoinositide-specific phospholipase C [33]; as a calcium-storage protein [34]; and as a molecular chaperone [35]. PDI has been localized in the lumen of the endoplasmic reticulum and in protein bodies [36]; (4) SAMS2 catalyzes the transfer of the adenosyl moiety of ATP to the sulfur atom of methionine, resulting in the formation of *S*-adenosyl-methionine (SAM). In plants, SAM functions as a methyl donor in the transmethylation of lignin, DNA and alkaloids. SAM also is an intermediate in the biosynthesis of polyamines and ethylene or serves as an allosteric activator of threonine synthase [37,38]; (5) FQR1 shares 64–84% identity with three *Arabidopsis* quinone reductases. FQR1 is a novel auxin-responsive enzyme that possibly acts in detoxification reactions by reducing highly reactive and potentially destructive semiquinones [39]. The plant derived enzyme reduces quinones and semiquinones caused by the delignification reactions of pathogens [40]; (6) cytosolic TPI catalyzes the isomerization of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate during both gluconeogenesis and glycolysis and is expressed ubiquitously. Transcript levels of TPI in rice were increased by submergence [41]. The only identified protein that was repressed by trehalose was Rubisco activase. Rubisco activase removes ribulose 1,5-bisphosphate and the nocturnal inhibitor, carboxyarabinitol 1-phosphate from the active site of Rubisco, i.e., ribulose 1,5-bisphosphate carboxylase/oxygenase [42]. Rubisco activase also performs an ATP-dependent reaction that facilitates carbamylation of Rubisco to form the catalytically active form of the enzyme [43]. Additionally, Rubisco activase was light activated by thioredoxin-*f* [44].

Two of the proteins induced by trehalose in *Arabidopsis*, SAMS2 and DHAR1 provide protection from abiotic or biotic stress. As noted above DHAR1 functions in ascorbate synthesis and is involved in controlling reactive oxygen species, which can proliferate during various plant stresses [45]. The fact that SAM is an intermediate in ethylene and polyamine synthesis also indicates a potential role for SAMS2 in stress responses. Moreover, two other proteins induced by trehalose may function in the removal of toxins from the cell. Living cells possess a three-phase system for the detoxification of harmful xenobiotic compounds [46]. Phase one enzymes, such as cytochrome P450, catalyze oxidation, reduction or hydrolysis reactions that introduce or expose functional groups on deleterious compounds. During the second phase of detoxification, metabolites produced during phase I are conjugated with sugars or with the tripeptide, GSH. In mammalian tissues, GSTs and QRs are classified as phase II detoxifying enzymes. Thus, the induction of FQR1 and GSTF2 in *Arabidopsis* was likely a detoxification response. In the third phase of detoxification, glycosylated or glutathione tagged molecules are recognized by an ATP-binding cassette (ABC), which serves as a membrane transporter on the tonoplast or plasma membrane [47]. Conjugates formed during phase II of detoxification are then exported into the vacuole or apoplast.

According to previous reports [13,48], sucrose added to the growth medium in the present study would be expected to induce and repress various plant genes. Sucrose was added to both the control and trehalose treated samples to facilitate plant growth. Only proteins that differed between the two treatments were identified by imaging silver-stained gels. This suggested that changes of protein levels were the result of altered gene expression in response to trehalose treatment. We cannot rule out the possibility that trehalose treatment modified changes of gene expression due to high sucrose levels in the growth medium. In support of this conclusion Wingler et al. [7] showed that trehalose induced β -amylase expression in *Arabidopsis* when sucrose was present but no change in gene expression occurred when trehalose was used alone.

In summary, trehalose metabolism is ubiquitous in higher plants and T6P is essential for specific aspects of plant growth including embryo maturation in *Arabidopsis*. In the current study seven proteins were identified that were altered by feeding *Arabidopsis* seedlings exogenous trehalose for 6–12 h that had not been identified in previous investigations. Four detoxification and stress response proteins were among the seven proteins induced by the trehalose treatment. Current findings extended previous results showing a relationship between exogenous trehalose and the induction of genes involved in plant responses to biotic and abiotic stress [8,18]. The exposure of *Arabidopsis* seedlings to trehalose resulted in the accumulation of disaccharides, possibly from the culture medium. We hypothesized that this subsequently altered polypeptide abundance via carbohydrate regulated gene expression. Since trehalose is produced

by various microbes and is present in the environment, it is not surprising that plants have developed defense mechanisms to counteract its toxic effects [14].

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